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Molecular Characterization of TEM-type β-Lactamases Identified in Cold-Seep Sediments of Edison Seamount (South of Lihir Island, Papua New Guinea)

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To determine the prevalence and genotypes of β-lactamases among clones of a metagenomic library from the cold-seep sediments of Edison seamount (10,000 years old), we performed pulse-field gel electrophoresis, antibiotic susceptibility testing, pI determination, and DNA sequencing analysis. Among the 8,823 clones of the library, thirty clones produced β-lactamases and had high levels of genetic diversity. Consistent with minimum inhibitory concentration patterns, we found that five (16.7%) of thirty clones produced an extended-spectrum β-lactamase. 837- and 259-bp fragments specific to *bla*$_{TEM}$ genes were amplified, as determined by banding patterns of PCR amplification with designed primers. TEM-1 was the most prevalent β-lactamase and conferred resistance to ampicillin, piperacillin, and cephalothin. TEM-116 had a spectrum that was extended to ceftazidime, cefotaxime, and aztreonam. The resistance levels conferred by the pre-antibiotic era alleles of TEM-type β-lactamases were essentially the same as the resistance levels conferred by the TEM-type alleles which had been isolated from clinically resistant strains of bacteria of the antibiotic era. Our first report on TEM-type β-lactamases of the pre-antibiotic era indicates that TEM-type β-lactamases paint a picture in which most of the diversity of the enzymes may not be the result of recent evolution, but that of ancient evolution.

Key words: β-lactamases, TEM-1, TEM-116, metagenomic library

The β-lactamases (EC 3.5.2.6) produced by bacteria are known to protect against the lethal effects of β-lactams (penicillins, cephalosporins, carbapenems, or monobactams) on cell wall synthesis. Among clinical isolates of the family *Enterobacteriaceae*, the production of β-lactamase is the single most prevalent mechanism responsible for resistance to β-lactams (Sanders and Sanders, 1992). A variety of β-lactamases has been classified into classes A, B, C, and D, according to their amino acid homology (Ambler, 1980). The most common β-lactamases among the *Enterobacteriaceae* are the plasmid-born class A TEM (named for a patient called Temoniera) and SHV (named for sulfhydryl variable) β-lactamases (Medeiros, 1997). First reported in 1965 (Datta and Kontomichalou, 1965), TEM-1 (classical TEM-type β-lactamase) confers a high level of resistance to penicillins (ampicillin and piperacillin) and early cephalosporins (cephalothin), but confers little resistance to oxyiminocephalosporins (cefotaxime and ceftazidime), carbapenems (imipenem and meropenem), and monobactams (aztreonam) (Medeiros, 1997). Beginning in the early 1980s and after the introduction of β-lactam antibiotics into medical use, extended-spectrum β-lactamases (ESBLs) driven from TEM-1 began to appear. ESBLs are β-lactamase inhibitor-susceptible enzymes which confer broad resistance to penicillins, aztreonam, cefotaxime, and ceftazidime (Livermore *et al.*, 1995). ESBLs are often plasmid-mediated, and most are mutants of TEM-1 enzymes, with one or more amino acid substitutions around the active site (Paterson *et al.*, 2001). These changes allow for the hydrolysis of extended-spectrum cephalosporins (ceftazidime and cefotaxime) and monobactams (aztreonam), which are stable to TEM-1 enzymes (DuBois *et al.*, 1995). The rapid appearance of new TEM variants active against those β-lactams has greatly affected public health policy and has conditioned thinking in regards to the evolution of antibiotic resistance. Hall and Barlow (2004) estimated that TEM β-lactamases diverged around 400

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million years ago (before the antibiotic era). Due to the lack of a fossil record, the values for the times of divergence are fairly speculative. Therefore, investigation of β-lactamase genes from microorganisms of the pre-antibiotic era is necessary. Deep-sea surface sediments harboring ancient microorganisms were estimated to range in age from 5800 to approximately 180,000 years (Raghukumar *et al*, 2004). A metagenome is defined as the collective genomes of all microorganisms in a given habitat. It may, therefore, act as the starting material for culture-independent microbial genomic analysis (Hendelsman *et al*, 1998). Thus, metagenomics can be used to address the challenge of studying microorganisms in an environment such as that of cold-seep sediments. These microorganisms are, as yet, unculturable and represent more than 99% of the organisms in the cold-seep sediment environment (Amann *et al.*, 1995).

In this study, we detected TEM-type β-lactamases from a metagenomic library of the cold-seep sediments of deep-sea Edison seamount (about 10,000 years old, Schmidt *et al*, 2002) and characterized these β-lactamases. We suggested that β-lactam resistance in microorganisms was likely to have been present prior to the modern antibiotic era, and paid particular attention to understanding the rapid appearance of strains that express β-lactamase genes subsequent to the introduction of β-lactams into medical and agricultural use.

Materials and Methods

Bacterial strains

A total of 8,823 clones of a metagenomic library were constructed. Thirty clones were selected as β-lactamase-producing clones. *E. coli* EPI 300 strain (Epicentre, Madison, USA) was used as a host of the metagenomic library construction. *E. coli* ATCC 25922 was used as the minimum inhibitory concentration (MIC) reference strain.

Metagenomic library construction

During the September 2002 research cruise, SO-166, cold-seep sediment (depth, 1,450 m; showing microbial diversity) samples were obtained at the summit of the deep-sea Edison seamount (03°19'S, 152°34'E), south of Lihir Island in the New Ireland Fore-arc, near Papua New Guinea, from the R/V Sonne. With a camera-guided grab sampler and the assistance of the captain of the R/V Sonne, P. M. Herzig, we obtained intact sediments from the inside of sediments. Our method of sampling allowed these sediments to remain devoid of contamination. The sediment samples were aliquoted into sterile conical tubes and preserved at 4°C during transportation for 2 weeks. They were then stored at -80°C. Community DNA was extracted from the sediment samples by a method which uses a cation-exchange resin (Hurt *et al*, 2001). The extracted DNA was end-repaired with end-repair enzyme mix (Epicentre) which made the DNA blunt-ended and 5-phosphorylated. The end-repaired DNA was separated by low-melting-temperature agarose gel (SeaPlaque, Cambrex, USA) electrophoresis at 35V for 13 h. The regions containing approximately 40 kb DNA fragments were cut from the gel. A metagenomic library was constructed using the approximately 40 kb end-repaired DNA fragments and CopyControl™ Fosmid Library Production Kit (Epicentre, USA). CopyControl cloning system was based on technology developed by Wild *et al.* (2002). The recombinant fosmid containing the end-repaired DNA fragments was purified by the alkaline lysis method (Sambrook and Russel, 2001).

Screening of β-lactamase-producing clones from the metagenomic library

In order to screen β-lactamase-producing clones from the metagenomic library, a total of 8,823 fosmid clones were transferred to Luria-Bertani (LB, Difco, USA) plates, each containing 100 μg/ml ampicillin. The ampicillin-resistant clones were selected for further analyses. Unless otherwise stated, molecular biological reagents and restriction enzymes were obtained from Sigma-Aldrich (USA). To test the genetic diversity of fosmid clones, the purified fosmid was digested with *Bam*HI and was analyzed by pulse-field gel electrophoresis (PFGE). *Bam*HI-digested fosmid DNA was prepared according to the instructions of Bio-Rad (USA) and fragments were separated for 12 h at 6 V/cm at 11°C using a CHEF-DRII system (Bio-Rad, USA), with initial and final switch times of 0.05 and 0.46 sec, respectively. DNA fingerprints were interpreted as recommended by Tenover *et al.* (1995).

Susceptibility to β-lactams

Minimum inhibitory concentrations (MICs) of antimicrobial agents were determined by the agar dilution method (Lee *et al.*, 2003). Antibiotics were produced by and obtained from the following suppliers: ampicillin and cephalothin (Sigma, USA); cefotetan (Merck Sharp and Dohme-Chibret, USA); cefotaxime (Handok Pharmaceuticals, Korea); ceftazidime and ampicillin-sulbactam (Hanmi Pharmaceuticals, Korea); aztreonam and cefepime (BMS Pharmaceutical Korea, Korea); cefoxitin, imipenem, and meropenem (Choongwae Pharmaceuticals, Korea); piperacillin (Yuhan, Korea); and piperacillin-tazobactam (Wyeth Korea, Korea). The results of antimicrobial susceptibility tests were interpreted according to the National Committee for Clinical Laboratory Standards' criteria (NCCLS, 2000).

Isoelectric focusing (IEF)

Crude cell extracts containing β-lactamases from fosmid clones were prepared by the osmotic shock method detailed in the pET system manual (Novagen, USA). IEF was performed in Ready Gel Precast IEF Polyacrylamide

Gel containing Ampholine with a pH range of 3.5 to 9.5, placed into a Mini-Protein 3 Cell, as described by the manufacturer (Bio-Rad, USA). Gels were developed with 0.5 mM nitrocefin (Oxoid, United Kingdom) as a chromogenic substrate (cephalosporin) of β-lactamases.

Molecular studies

The purified fosmid DNA was used as template DNA in polymerase chain reaction (PCR). Using the Primer Calculator program (Williamstone Enterprises, USA), the primers for PCR amplification were designed by selecting consensus sequences in multiple-nucleotide alignment of 60 TEM-type β-lactamase genes (bla_{TEM}), 27 SHV-type β-lactamase genes (bla_{SHV}), and 5 CMY-type β-lactamase (class C) genes (bla_{CMY}). The primers were described previously (Lee *et al.*, 2000): T1, T2, T3, and T4 were used for bla_{TEM}; S1, S2, S3, and S4 were used for bla_{SHV}; C1, C2, C3, and C4 were used for bla_{CMY}. PCR amplifications were carried out on a GeneAmp® PCR System 2400 (Perkin-Elmer Cetus, USA) with Premix *Taq* kit containing TaKaRa Ex *Taq* polymerase (TaKaRa, Japan). The composition of the reaction mixture was as follows: 25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, deoxynucleotide triphosphates (0.2 mM each), and 1.2 U of TaKaRa Ex *Taq* polymerase in a total volume of 49 μl. A total of 1 μl (5 ng) of template DNA was added to the reaction mixture, which was briefly centrifuged and stored at 4°C for 30 min. The PCR program consisted of an initial denaturation step at 96°C for 30 sec, followed by 30 cycles of DNA denaturation at 96°C for 30 sec, primer annealing at 50 ~ 60°C (50°C for bla_{TEM}, 56°C for bla_{SHV}, 60°C for bla_{CMY}) for 30 sec, and primer extension at 72°C for 10 min. DNA sequencing was performed by the direct sequencing method with an automatic sequencer (ABI PRISM3100; Applied Biosystems, Germany), as previously described (Lee *et al.*, 2001).

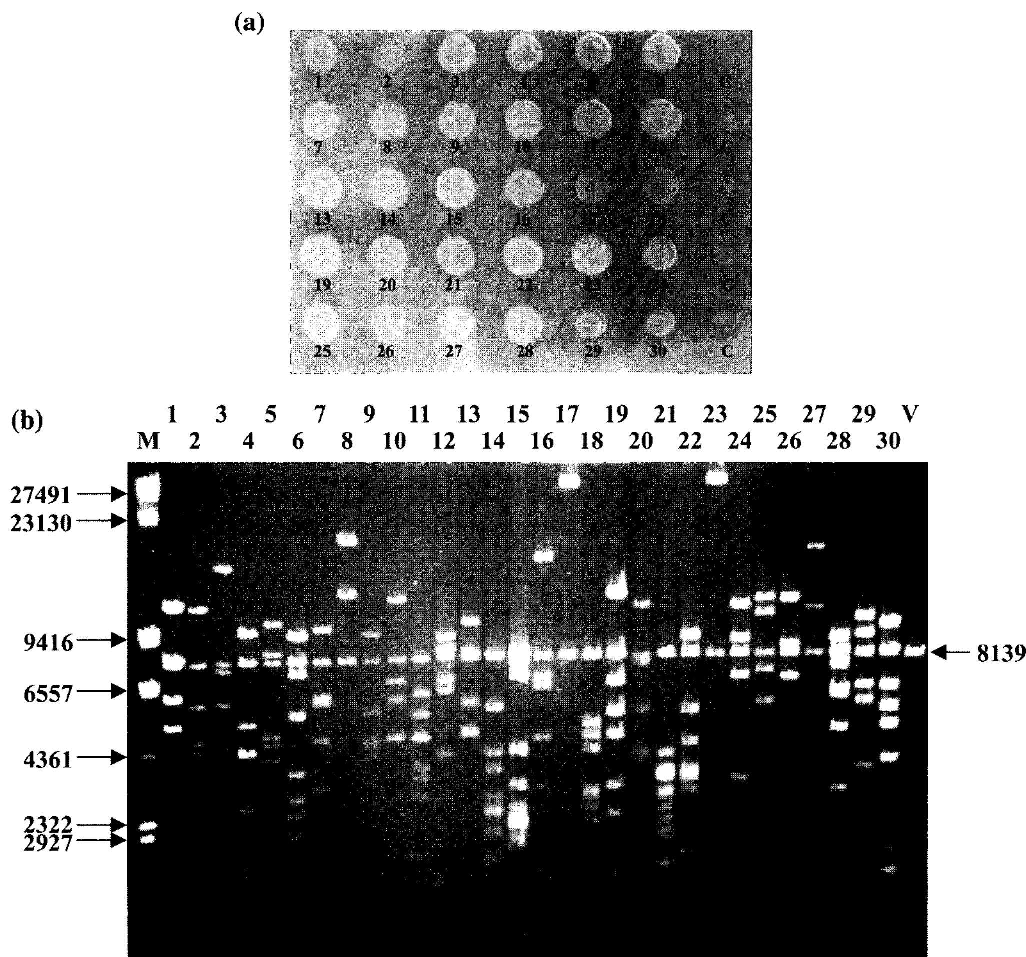

Fig. 1. Restriction analysis (b) by *Bam*HI of thirty clones (a) producing β-lactamase. Thirty clones (a) grew on LB plates containing 100 μg/ml ampicillin, but negative controls (*E. coli* EPI 300 host strain, C) did not grow. Each number of (a) indicates each clone identical to that of (b). Lanes of (b): M, λ *Hin*dIII moleculars mass markers [sizes in base pairs (bp) are indicated on the left edge of the gel]; V, Fosmid vector pCC1FOS™ (8139 bp); 1, ES01003A02; 2, ES01003G06; 3, ES01008A11; 4, ES01008C04; 5, ES01008E12; 6, ES01008F07; 7, ES01013B05; 8, ES01013E09; 9 ES01013F06; 10, ES01014A10; 11, ES01014D12; 12, ES01014G12; 13, ES01016A08; 14, ES01016A11; 15, ES01016B02; 16, ES01016C11; 17, ES01019B07; 18, ES01020A11; 19, ES01023A03; 20, ES01023A09; 21, ES01023B06; 22, ES01023H02; 23, ES01023H07; 24, ES01024D03; 25, ES01024E08; 26, ES01027F09; 27, ES01031E08; 28, ES01031F09; 29, ES01033B01; 30, ES01046B06.

DNA sequence analysis was performed with DNASIS for Windows (Hitachi Software Engineering America Ltd., USA). Database similarity searches for both the nucleotide sequences and deduced protein sequences were performed with BLAST at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Results

Construction of a metagenomic library

The environmental DNA from sediments of the deep-sea Edison seamount was used to construct a metagenomic library using the fosmid vector, pCC1FOS (Epicentre). ES01 was the first metagenomic library, consisting of 8,823 clones and stored at -80 in 96-well microplates. The average insert DNA size was 32.3 kb (Fig. 1b). It was estimated that the library ES01 contained approximately 284 Mbp of DNA, which corresponded to about 95 bacterial genomes based on the average bacterial genome of 3 Mbp.

Screening of β-lactamase-producing clones

Thirty of 8,823 clones grew on an LB plate containing 100 μg/ml ampicillin (Fig. 1a). The cell extracts of thirty

Table 1. Profiles of β-lactamase-producing clones, MIC reference strain (*E. coli* ATCC 259220), and host strain (*E. coli* EPI 300) for transfection

Clones or strains	MIC(μg/ml) of the following β-lactams[a]:													pI	β-lactamase
	AMP	A/S	PIP	P/T	CEP	FOX	CTT	CAZ	CTX	FEP	IPM	MER	AZT		
ES01003A02	256	8	>256	16	16	0.25	0.25	0.12	0.12	0.12	0.25	0.12	0.50	5.4	TEM-1
ES01003G06	256	16	>256	16	32	0.50	0.25	0.50	0.25	0.25	0.12	0.12	0.50	5.4	TEM-1
ES01008A11	128	16	>256	32	16	0.25	0.25	0.50	0.25	0.25	0.25	0.25	0.50	5.4	TEM-1
ES01008C04	256	16	>256	32	32	0.50	0.50	0.50	0.25	0.25	0.12	0.12	0.25	5.4	TEM-1
ES01008E12	256	8	>256	16	32	0.25	0.50	0.12	0.25	0.12	0.25	0.12	0.50	5.4	TEM-1
ES01008F07	>256	16	>256	32	128	0.50	0.50	64.00	64.00	0.5	0.25	0.12	32.00	5.4	TEM-116
ES01013B05	256	16	>256	32	32	0.50	0.50	0.50	0.25	0.25	0.12	0.12	0.25	5.4	TEM-1
ES01013E09	>256	8	>256	32	128	0.50	0.25	64.00	64.00	0.25	0.25	0.12	32.00	5.4	TEM-116
ES01013F06	256	8	>256	16	32	0.25	0.50	0.25	0.25	0.25	0.25	0.12	0.25	5.4	TEM-1
ES01014A10	256	8	>256	32	32	0.50	0.50	0.25	0.25	0.25	0.25	0.12	0.25	5.4	TEM-1
ES01014D12	256	16	>256	32	32	0.50	0.25	0.25	0.25	0.12	0.12	0.12	0.50	5.4	TEM-1
ES01014G12	128	8	>256	16	16	0.50	0.12	0.25	0.25	0.12	0.25	0.25	0.25	5.4	TEM-1
ES01016A08	256	8	>256	32	16	0.50	0.25	0.25	0.25	0.12	0.25	0.12	0.25	5.4	TEM-1
ES01016A11	>256	16	>256	32	64	0.50	0.50	32.00	64.00	0.50	0.25	0.12	32.00	5.4	TEM-116
ES01016B02	256	16	>256	16	16	0.50	0.25	0.50	0.50	0.50	0.12	0.12	0.25	5.4	TEM-1
ES01016C11	256	8	>256	16	32	0.50	0.50	0.25	0.25	0.25	0.25	0.25	0.25	5.4	TEM-1
ES01019B07	256	8	>256	32	16	0.50	0.25	0.50	0.50	0.25	0.25	0.25	0.25	5.4	TEM-1
ES01020A11	256	8	>256	32	32	0.50	0.25	0.25	0.50	0.25	0.25	0.12	0.25	5.4	TEM-1
ES01023A03	256	8	>256	16	32	0.25	0.50	0.25	0.25	0.25	0.25	0.12	0.25	5.4	TEM-1
ES01023A09	>256	16	>256	32	64	0.25	0.25	32.00	64.00	0.25	0.50	0.25	32.00	5.4	TEM-116
ES01023B06	128	8	>256	16	16	0.50	0.25	0.25	0.50	0.12	0.25	0.12	0.50	5.4	TEM-1
ES01023H02	256	16	>256	32	16	0.50	0.50	0.50	0.50	0.25	0.25	0.06	0.25	5.4	TEM-1
ES01023H07	256	16	>256	32	16	0.25	0.50	0.50	0.50	0.50	0.06	0.06	0.25	5.4	TEM-1
ES01024D03	256	8	>256	16	32	0.25	0.25	0.50	0.25	0.12	0.12	0.12	0.25	5.4	TEM-1
ES01024E08	256	16	>256	16	32	0.25	0.25	0.50	0.25	0.25	0.25	0.12	0.25	5.4	TEM-1
ES01027F09	>256	16	>256	32	128	0.50	0.25	64.00	64.00	0.50	0.12	0.25	32.00	5.4	TEM-116
ES01031E08	128	4	>256	16	16	0.50	0.50	0.25	0.25	0.12	0.25	0.12	0.50	5.4	TEM-1
ES01031F09	256	8	>256	16	32	0.50	0.50	0.25	0.25	0.25	0.12	0.12	0.50	5.4	TEM-1
ES01033B01	256	16	>256	16	32	0.25	0.25	0.50	0.25	0.25	0.25	0.25	0.25	5.4	TEM-1
ES01046B06	256	8	>256	16	16	0.25	0.12	0.25	0.25	0.12	0.25	0.12	0.25	5.4	TEM-1
ATCC 25922	0.5	0.5	0.5	0.5	0.5	0.12	0.12	0.12	0.25	0.12	0.06	0.06	0.12		
E. coli EPI 300	0.5	0.5	0.5	0.5	0.5	0.12	0.12	0.12	0.12	0.12	0.06	0.06	0.12		

[a]Abbreviation: AMP, ampicillin; A/S, ampicillin/sulbactam; PIP, piperacillin; P/T, piperacillin/tazobactam; CEP, cephalothin; FOX, cefoxitin; CTT, cefotetan; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MER, meropenem; AZT, aztreonam.

clones changed the color from the pale yellow of nitrocefin to the pink of cephalosporanic acid (the end product hydrolyzed from nitrocefin by β-lactamases), which indicated that all thirty clones were β-lactamase producers. The isoelectric point (pI) value of β-lactamases produced by the thirty clones was 5.4 (Table 1).

PFGE analysis generates a characteristic genomic fingerprinting which can be used to reveal intra- and interspecies genotypic variations. All thirty clones generated distinct bands by *Bam*HI macrorestriction analysis, ranging in size from 0.5 to 28.1 kb and varied from 3 to 11 bands (Fig. 1b). Distinct PFGE patterns indicated that all thirty clones showed different genotypes.

Susceptibility to β-lactams

β-lactam MICs and pI for thirty clones are listed in Table 1. As indicated by the National Committee for Clinical Laboratory Standards' criteria, all clones were resistant to ampicillin, piperacillin, and cephalothin, but were sensitive to cefoxitin, cefotetan, cefepime, imipenem, and meropenem. All clones showed from a sixteen-fold to a greater than thirty two-fold decrease in the MIC of ampicillin and piperacillin by β-lactamase inhibitors (sulbactam and tazobactam, respectively). Five clones (ES01008F07, ES01013E09, ES01016A11, ES01023A09, and ES01027F09) were resistant to ceftazidime, cefotaxime, and aztreonam. The resistance phenotypes of these five clones indicated that they produced ESBL(s). All clones produced β-lactamases with a pI of 5.4.

Genotyping of β-lactamases

Taking into consideration the resistance phenotypes of these thirty clones, the resistance genotypes of these clones were analysed. Using the designed primers, we detected β-lactamase genes by PCR. The electrophoretic analysis of PCR product showed that TEM-type β-lactamases genes (bla_{TEM}) were accurately amplified and the fragment sizes of PCR product were the same as indicated in Materials and Methods (Fig. 2). SHV-type β-lactamase genes and CMY-type β-lactamase genes were not detected. The resistance genotypes of thirty clones were analyzed by direct sequencing of the PCR-amplified bla_{TEM} genes. Only one large open reading frame was found, which corresponds to a putative protein of 286 amino acids for TEM-type β-lactamase. Two different TEM-type (bla_{TEM-1} and $bla_{TEM-116}$) β-lactamase sequences were found (Table 1). Table 1 showed that five clones (ES01008F07, ES01013E09, ES01016A11, ES01023A09, and ES01027F09) among thirty clones produced TEM-116. The remaining twenty-five clones produced TEM-1.

Discussion

Traditional methods of culturing microorganisms that

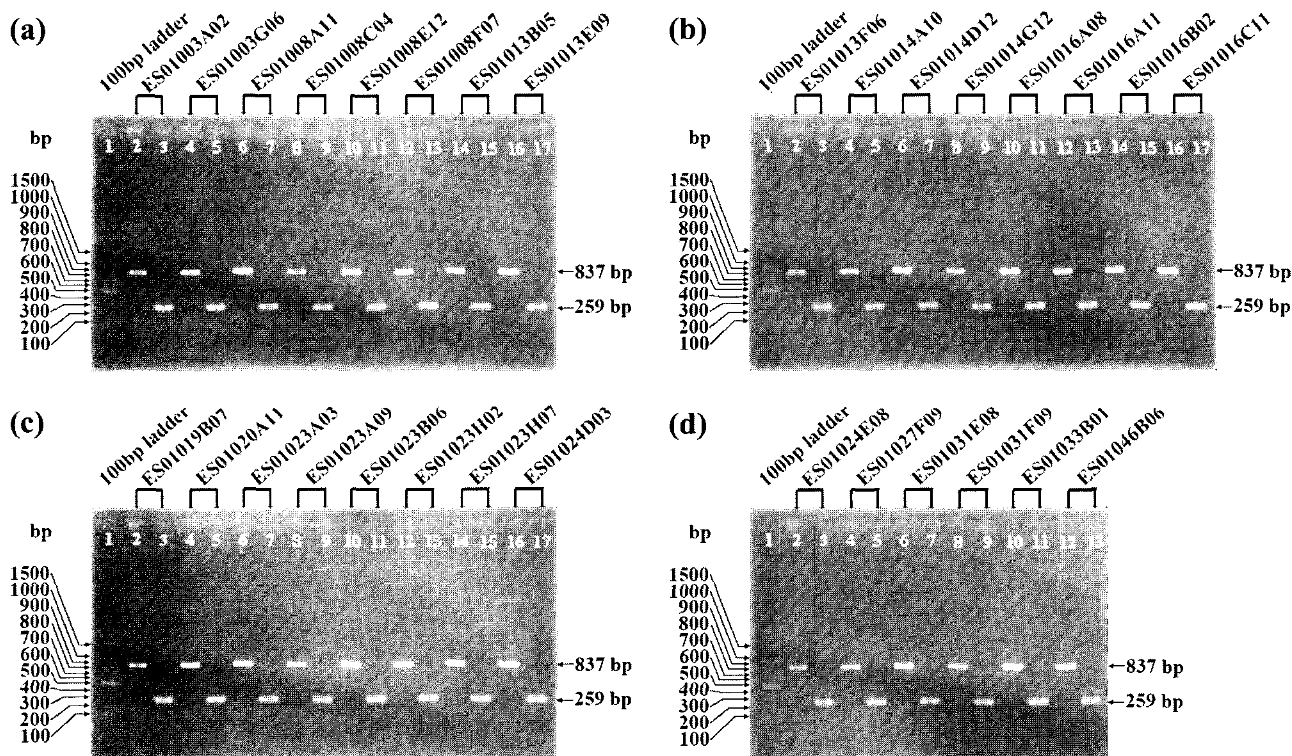

Fig. 2. Banding patterns of PCR products of bla_{TEM} β-lactamase genes. Lane 1 of each figure: DNA molecular mass markers [100 bp ladder, sizes in base pairs (bp) are indicated on the left edge of the gel]. Even lanes in (a), (b), (c), and (d): PCR products (837 bp) of bla_{TEM} with T1 and T2 primer pair. Odd lanes in (a), (b), (c), and (d): PCR products (259 bp) of bla_{TEM} with T3 and T4 primer pair.

grow under laboratory conditions limit the analysis of true microbial diversity and function (Amann *et al.*, 1995; Hugenholtz *et al.*, 1996). In order to investigate β-lactamases from an ancient habitat, we constructed a metagenomic library (ES01) from the cold-seep sediments of the deep-sea Edison seamount (about 10,000 years old, Schmidt *et al*, 2002). PFGE analysis of thirty clones producing β-lactamases from the ES01 metagenomic library indicated that they were not closely related, but had a high level of genetic diversity. Our partial results of the end-sequencing of the ES01 library and previous data of microbial diversity in the cold-seep sediments of Edison seamount (Lee *et al.*, 2004) showed that microorganisms identified in the sediments were primarily affiliated with one of four groups: the λ-, δ-, and ε-subdivisions of *Proteobacteria*, and *Cytophaga-Flavobacterium-Bacteroides*.

On the basis of the pI of β-lactamases, their resistance phenotypes against β-lactams, their profiles of inhibition by sulbactam and tazobactam, and DNA sequencing of PCR-amplified bla_{TEM} genes, the β-lactamase produced by five clones (ES01008F07, ES01013E09, ES01016A11, ES01023A09, and ES01027F09) was a TEM-type ESBL (TEM-116). The β-lactamase produced by the remaining twenty-five clones was a classical TEM-type β-lactamase (TEM-1). These β-lactamases had the same MIC patterns against β-lactams, amino acid sequences, and pI as those previously reported in clinical isolates (Giakkoupi *et al.*, 2000; Jeong *et al.*, 2004). The prevalences of TEM-1 and TEM-116 in the sediments of Edison seamount were 0.3% (25 of 8,823) and 0.06% (5 of 8,823), respectively. Across the globe, TEM-1 β-lactamase exists at high frequencies in antibiotic-resistant pathogens (Medeiros, 1997; Yan *et al*, 2000). While TEM-1 has a spectrum that is limited to penicillins and early cephalosporins, it has given rise to more than ninety descendent alleles, such as TEM-116, that confer resistance to most modern β-lactam antibiotics (the website of The Hall Laboratory of Experimental Evolution, http://www.rochester.edu/Colledge/BIO/labs/HallLab/AmpC-Phylo.html). The deduced amino acid sequence of TEM-116 had two amino acid substitutions from TEM-1 (Table 2). Two schemes for in vivo evolution were reported: (i) from TEM-1 to TEM-19, from TEM-19 to TEM-20, and from TEM-20 to TEM-52 and (ii) from TEM-1 to TEM-116 (Jeong *et al.*, 2004). The TEM descendants might have evolved from TEM-1 after the beginning of the antibiotic era, in approximately 1950. However, TEM-116 and TEM-1 β-lactamase genes were both detected in sediments of the deep-sea Edison seamount, which is estimated to be approximately 10,000 years old (Schmidt *et al*, 2002). Therefore, the hypothesis that antibiotic resistance genes have evolved exclusively in response to the modern clinical use of antibiotics, such as β-lactams, can be plausibly disputed. There is growing evidence that resistance genes were capable of conferring resistance long before the discovery and use of modern antibiotics. An example of this evidence exists in the two strains of *Citrobacter freundii* that were collected prior to the dawn of the antibiotic era (the 1920s) which carried *ampC* β-lactamase genes whose products were entirely as active toward as wide a range of β-lactams as were the AmpC β-lactamases (class C) that were found on plasmids after the antibiotic era began (Barlow and Hall, 2002). Although the reason that only TEM-type β-lactamase genes were detected in the sediments was not clear, it might be supposed that there were primarily *γ-Proteobacteria* (mostly detected in the sediments) harboring only TEM-type β-lactamase genes and not SHV-type, CMY-type, etc. Our first report on the TEM-type β-lactamases (class A) which existed prior to the antibiotic era indicates that TEM-type β-lactamases paint a picture in which most of the diversity of the enzymes may be the result of ancient evolution. According to these results and because classical β-lactamases and ESBLs were present long before the antibiotic era began, we should not be surprised by the rapid appearance of strains that express those genes subsequent to the introduction of β-lactam antibiotics into medical and agricultural use.

Table 2. Amino acid substitutions of TEM-1 and TEM-116 β-lactamase

β-lactamase	pI	Residue (coding triplet) at amino acid: 84	184
TEM-1	5.4	Val (GTT)	Ala (GCA)
TEM-116	5.4	Ile (ATT)	Val (GTA)

Acknowledgments

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